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Acetylcholinesterase (AChE) Insensitivity of *Aedes aegypti* Exposured by Citronella Oil (*Cymbopogon nardus*)

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ABSTRACT

Aedes mosquitoes are vectors of viruses, namely the dengue virus. Treatment with insecticides is no longer effective if the target mosquito is resistant. Therefore, efforts are needed to overcome the resistance of these mosquitoes. Inhibition of the enzyme acetylcholinesterase (AChE) is one way the synthetic insecticide Profenofos works; if AChE is inhibited, acetylcholine accumulation will disrupt the nervous system. Insensitivity to AChE is one mechanism of mosquito resistance to insecticides. Based on this, the problem is formulated as follows: is the increase in susceptibility of *Aedes aegypti* mosquitoes to alphacymethrin, citronella oil, and a combination of both caused by a decrease in the insensitivity of mosquito AChE? This study aims to determine the AChE insensitivity of *Aedes aegypti* mosquitoes to citronella oil, a combination of citronella oil and alphacymethrin 0.03%), compared to the positive control (insecticide alphacymethrin 0.03%). Percentage of mosquito mortality after being held for 24 hours: The results showed no difference between the two groups of mosquitoes without treatment (A) and 100 µL/L citronella oil (B), F count 0.000, Sig. 1.000, significance value <0.05. Exposure to 100 µL/L citronella oil on instar III-IV mosquito larvae did not make mosquitoes more susceptible or more resistant to 0.03% alphacypermethrin insecticide. Based on the absorption value analysis results using the T-test between group A/ without treatment and group B with 100µL/L citronella oil treatment showed a Sig. (2-tailed) value of 0.547>0.05 means there is no significant difference between the two groups.

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INTRODUCTION

Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) have become international public health problems (WHO, 2011). DHF is caused by a Flaviviradae family virus spread by the Aedes mosquito (Stegomyia). There is no specific treatment for dengue fever, but appropriate medical care often saves the lives of patients with severe dengue hemorrhagic fever. The most effective way to prevent dengue virus transmission is to fight mosquitoes that carry the disease (WHO, 2011). Using insecticides is one effort to control mosquitoes that carry this disease. Insecticides can control insects by disrupting or damaging the systems in the insect's body. Generally, currently 4 (four) groups are used, namely organochlorines, organophosphates, carbonates, and pyrethroids. Cypermethrin is a class of pyrethroid insecticides that people often use against Aedes mosquitoes because the price is affordable (Ministry of Health Republic Indonesia, 2012). New problems arise when insect resistance to insecticides occurs due to long-term exposure. The *Aedes aegypti* mosquito can develop an immune system against the insecticides used. Resistant insects will reproduce, and the genetic changes will reduce resistant offspring, increasing the proportion of resistant vectors in the population (Ministry of Health Republic Indonesia, 2012). A study by Widiarti et al. (2011), the resistance of the *Aedes aegypti* mosquito to the insecticides malathion 0.8%, Bendioc arb 0.1%, Landasihalothrin 0.05%, permethrin 0.75%, deltamethrin 0.05% and Etofenproks 0, 5% occurred in Central Java and the unique region of Yogyakarta. Resistance also occurred in Cimahi City, West Java (Wahyudin & Mulyaningsih, 2009), which tested the susceptibility of the *Aedes aegypti* mosquito to several insecticides. The results were that *Aedes aegypti* was resistant to organophosphate insecticides, tolerant to pyrethroids, and resistant to cypermethrin 0.2% and 0.4% (Pradani et al., 2011). Controlling insecticides is no longer practical if the target insect is resistant. So, efforts are needed to overcome the resistance of the targeted insects. Citronella can be used as a natural pesticide. The leaves and stems are distilled to produce an essential oil known as citronella. People use lemongrass oil to repel mosquitoes and other insects by rubbing it on their skin or clothes (Puslitbang, 2012). Wahyuningtyas (2004) stated that citronella at a concentration of 2.5% could repel the *Aedes aegypti* L mosquito. Khoiriyah and Nurminha (2021) conducted a study on the resistance of Aedes aegypti mosquitoes exposed to citronella oil (*Cymbopogon nardus*); the results showed that the mosquitoes were resistant to 0.03% alphacypermethrin with a mortality percentage of 73.7%. However, they are susceptible to 10% citronella oil with a mosquito death percentage of 100%. Exposure to a combination of the insecticide alphacypermethrin and 10% citronella oil on mosquitoes increased mosquito susceptibility, as evidenced by an increase in the percentage of mosquito deaths.

Insects have an enzymatic system that neutralizes insecticide toxins. Dengue vector mosquitoes resist insecticides through two mechanisms: increased esterase enzyme activity and insensitivity to acetylcholine esterase (AChE) (Widiarti et al., 2011). The synthetic insecticide profenofos has one way of working, namely disrupting the insect's nervous system by inhibiting the action of the acetylcholinesterase (AChE) enzyme and causing the accumulation of acetylcholine (Untung, 2001; Djojosumarto, 2008). Mutchler (2010) stated that the accumulation of acetylcholine caused central nervous system disorders, seizures, respiratory paralysis, and death. Based on this, a study to determine the AChE insensitivity of *Aedes aegypti* mosquitoes to citronella oil (a combination of citronella oil and alphacypermethrin 0.03%) compared to the positive control (alphacypermethrin 0.03% insecticide) is necessary.

Based on this, the formulated problem is, namely, whether the increase in the susceptibility of the *Aedes aegypti* mosquito to alphacypermethrin, citronella oil, and the combination of the two is caused by a decrease in the mosquito's AChE insensitivity. This study aims to determine the AChE insensitivity of *Aedes aegypti* mosquitoes to citronella oil, a combination of citronella oil and alphacypermethrin 0.03%) compared to the positive control (alphacypermethrin 0.03% insecticide).

METHOD

The type of research is experimental research with a post-test design with the control group. The study was conducted at Balitbangkes Salatiga for bioassay tests and at the Tropical Medicine Laboratory, UGM Faculty of Medicine, for AChE insensitivity tests. The *Aedes aegypti* mosquito used as a sample was the *Aedes aegypti* mosquito resulting from colonization at the Salatiga Balitbangkes Entomology Laboratory, which was tested for resistance to the insecticide Alphacypermethrin 0.03%. Citronella oil (*Cymbopogon nardus* L.) Natuna Essential Merck. 10mL Origin: Sri Lanka, certified organic 100% Pure essential oil, which has been detected for citronella oil using Thin Layer Chromatography. Alphacymethrin 0.03% WHO Standard and PY Control WHO Standard. The research implementation consisted of 3 main parts, namely, detection of citronella oil compound, exposure of citronella to larvae to be colonized, susceptibility testing using the standard method (WHO, 2013) toward *Aedes aegypti* mosquitoes resulting from colonization with the following types of exposure: alphacypermethrin 0.03%, citronella oil, a combination of citronella oil and alphacypermethrin, as well as the acetylcholinesterase (AChE) insensitivity/insensitivity test using the Hemingway method on larvae and adult mosquitoes.

The ethical feasibility test was conducted at the Health Research Ethics Commission, Poltekkes Kemenkes Tanjungkarang, and was declared ethically feasible with letter number 113/KEPK-TJK/X/2022.

Analyze the significant differences between the three groups using ANOVA and continue with a minor real difference test to determine which groups are significantly different.

RESULTS

Detection of citronella essential oil

Citronella oil was detected for the presence of citronella essential oil using Thin-Layer Chromatography (TLC). Two (2) spots were obtained with Rf values of 0.05 and 0.68 on the TLC profile of the essential oil of Citronella

(*Cymbopogon nardus* L.); observations in UV_{254} light are shown in Table 1.

Table 1. Thin layer chromatography visualization of citronella essential oil (*Cymbopogon nardus* L.)

Sample	Spots			Rf value
	Visible	UV_{254}	UV_{366}	
Citronella	-	v	-	0,55
oil	-	v	-	0,68

Test for determining the concentration of citronella

Aedes aegypti larvae instar III-IV (L4) were exposed to citronella at 25, 50, 100, and 200µL/L concentrations. The control group consisted of 2 (two), namely a group without treatment and a control group with tween 80 (200µL/L). Exposure to the control tween 80 and citronella was carried out for 5 (five) minutes, and the live larvae were transferred to a container containing distilled water; after 24 hours, the number of dead larvae was counted. The number of larvae tested was 20 per replication. Each type of treatment consists of 3 (three) replications. The concentration of citronella upon exposure to larvae that colonized adult mosquitoes was determined based on the number of larval deaths in the treatment group. This concentration is the highest concentration of citronella that does not cause larval death. The definition of 0 (zero) larval death is that the larvae do not die after exposure to the citronella solution for 5 minutes and can survive 24 hours after being transferred to a tray containing distilled water (Table 2).

Table 2. Death number of *Aedes aegypti* larvae with exposure to citronella oil

Group	Citronella oil concentration	Replication	Death number of *Aedes aegypti* larvae	
			5 Minutes	24 hours
Group I	25 µL/L	1	0	0
		2	0	0
		3	0	0
Group II	50 µL/L	1	0	0
		2	0	0
		3	0	0
Group III	100 µL/L	1	0	0
		2	0	0
		3	0	0
Group IV	200 µL/L	1	0	1
		2	0	0
		3	0	0
Control with Tween 80 (200 µL/L)	-	1	0	0
		2	0	0
		3	0	0
Control without treatment	-	1	0	0
		2	0	0
		3	0	0

***Aedes aegypti* mosquito susceptibility test**

The results of continuous exposure to insecticides from generation to generation (F0-F20) caused mosquitoes to become tolerant to the insecticides. It was concluded that there was a change in the degree of susceptibility of the *Aedes aegypti* mosquito (Gunandini & Wicaksana, 2005). Mosquito susceptibility can be measured by carrying out a susceptibility test. Larvae that had been exposed to citronella at a concentration of 100µL/L for 5 minutes were transferred into a tray containing 1000mL of clear water; after 24 hours, the surviving larvae were reared/colonized into adult mosquitoes until the number of mosquitoes was sufficient to be tested for susceptibility (Tables 3 and 4).

Table 3 shows the number of mosquitoes that experienced knockdown during 60 minutes of observation. A mosquito from group A (without treatment) experienced knockdown at 60 minutes after being exposed to test paper containing solvent/ PY control/ negative control (PY Control). However, after holding for 24 hours, the results showed no deaths in this group. Groups A and B were exposed to alphacypermethrin 0.03% (positive control), 2.5% citronella oil, and a combination of 0.03% alphacypermethrin and 2.5% citronella oil showed all mosquitoes were knocked down at 60 minutes. All mosquitoes were declared dead after holding for 24 hours in all groups and treatments.

Table 3. ***Aedes aegypti*** **mosquito susceptibility test**

Adult mosquito groups	Interventions	%Knockdown 15 minutes	30 minutes	45 minutes	60 minutes	%Deaths 24 hours
Without Treatment (Group A)	Control PY	0	0	0	1,6	0
	Alpha-cypermethrin 0,03%	99,2	100	100	100	100
	Citronella oil 2,5%	72	98,4	100	100	100
	Combination of Alpha-cypermethrin 0,03% & Citronella oil 2,5%	100	100	100	100	100
Citronella oil 100µL/L (Group B)	Control PY	0	0	0	0	0
	Alpha-cypermethrin 0,03%	92	100	100	100	100
	Citronella oil 2,5%	80,8	96	97,6	100	100
	Combination of Alpha-cypermethrin 0,03% & Citronella oil 2,5%	94,4	100	100	100	100

However, after analyzing the data, a number of mosquitoes experienced knockdown and death. Table 4 shows that the PY Control intervention significantly differs from the other groups. The mosquito group exposed to 0.03% alphacypermethrin was substantially different from the 2.5% citronella oil group and not significantly different from those exposed to the combination of 0.03% alphacypermethrin and 2.5% citronella oil.

Table 4. Significant difference test in % knockdown between treatment groups

Intervention (I)	Intervention (J)	Mean Difference (I-J)	Std. Error	Sig.
PY Control	Alpha-cypermethrin 0,03%	-98.7000*	2.8042	.000
	Citronella oil 2,5%	-92.9000*	2.8042	.000
	Combination of alphacypermethrin 0,03% and citronella oil 2,5%	-99.1000*	2.8042	.000
Alpha-cypermethrin 0,03%	PY Control	98.7000*	2.8042	.000
	Citronella oil 2,5%	5.8000*	2.8042	.048
	Combination of alphacypermethrin 0,03% and citronella oil 2,5%	-.4000	2.8042	.888
Citronella oil 2,5%	PY Control	92.9000*	2.8042	.000
	Alphacypermethrin 0,03%	-5.8000*	2.8042	.480
	Combination of alphacypermethrin 0,03% and citronella oil 2,5%	-6.2000*	2.8042	.035
Combination of alpha-cypermethrin 0,03% and citronella oil 2,5%	PY Control	99.1000*	2.8042	.000
	Alpha-cypermethrin 0,03%	.4000	2.8042	.888
	Citronella oil 2,5%	6.2000*	2.8042	.035

Acetylcholinesterase (AChE) activity test

The acetylcholinesterase enzyme activity test was conducted on Aedes aegypti larvae and mosquitoes. This test was carried out by measuring the absorption value (AV) using an ELISA reader at a wavelength of 415 nm. The amount of absorption value (AV) is linear with the activity of the acetylcholinesterase enzyme. Tables 5 and 6 show the absorption values (AV) and analysis results.

Table 5 shows the highest average absorbance value in the group without treatment of 0.45550. The absorbance value of the citronella treatment group increased along with increasing citronella concentration. The p-value of $0.002 < 0.05$ indicates a real difference in the absorption value between the treatment groups.

Table 5. **Absorbance Value of *Aedes aegypti* larvae with citronella oil intervention**

Group	Absorbance Value (AV)		F	p-value
	Mean	SD		
Intervention I Citronella Oil 25µL/L	0.25538	0.094673	4.229	0.002
Intervention II Citronella Oil 50µL/L	0.28269	0.157000		
Intervention III Citronella Oil 100µL/L	0.31175	0.127248		
Intervention IV Citronella Oil 200µL/L	0.34763	0.111049		
Control with Tween 80 (200µL/L)	0.23175	0.054858		
Control Without Treatment	0.45550	0.118650		

Table 6 shows that mosquitoes raised from larvae exposed to citronella oil have lower absorbance values than mosquitoes raised from larvae without treatment. The data analysis showed no difference between the two groups, with a p-value of 0.547> 0.05.

Table 6. Absorbance Value (AV) of *Aedes aegypti* mosquitos

Adult mosquito groups	Mean±SD	t	Sig. (2-tailed)
Group A (without treatment)	1.361854±0.1458503	.616	.547
Group B (Citronella oil 100µL/L)	1.302042±0.3032056		

DISCUSSION

The essential oil in the citronella is known as citronella oil, whose components consist of alpha-citral, beta-citral, and nerol acetate. Detection is done to see the spots appearing on the TLC plate using UV254 light. The Rf value of citronella essential oil compounds (alpha-citral, beta-citral, nerol acetate) is ±0.694 (Astuti et al., 2012). Table 1 shows the results of TLC visualization with UV254; there are 2 (two) spots with Rf values of 0.55 and 0.68. This indicates that the citronella oil contains a citronella essential oil compound.

The concentration of citronella upon exposure to larvae, which would be raised as adult mosquitoes, was determined based on the number of larval deaths in the treatment group. This concentration is the highest concentration of citronella that does not cause larval death.

Table 2 shows larval deaths in the citronella treatment with a concentration of 200µL/L in 24-hour observations; the number of mortality was 1 (one) larva. All Aedes aegypti mosquito larvae lived in the control group, treated with citronella oil concentrations of 25, 50, and 100µL/L. So, the citronella concentration of 100µL/L was used as the intervention concentration for the citronella group of larvae that were raised into adult mosquitoes. The larval death rate of 0 (zero) is the expected larval death rate, so the concentration of citronella is on a microliter scale. The concentration of citronella was determined based on research of Gunandini and Wicaksana (2005) about the increase and activity of the acetylcholinesterase enzyme in Aedes aegypti mosquitoes selected with malathion. Intervention to this insecticide starts from the F0-F20 generation, concentrations 25, 50, 100, and 200µL/L, contact time 5 and 10 minutes. The study by Farich et al. (2021) showed that the LC50 of citronella essential oil was 1,533mg/L (1,533 ppm) for IV instar larvae and 11,804mg/L (11,804 ppm) for III instar larvae. Manimaran et al. (2012) conducted a citronella oil larvicidal test and obtained an LC50 of 47.21 ppm towards IV-instar larvae. The same research by Hazarika et al. (2018) showed different results, such as the LC50 value of citronella oil being 38.37 ppm when testing *Aedes aegypti* instar IV larvae.

Results Continuous exposure to insecticides from generation to generation (F0-F20) causes mosquitoes to become tolerant to the insecticides given; it is concluded that there is a change in the degree of susceptibility of the Aedes aegypti mosquito (Gunandini & Wicaksana, 2005). Mosquito susceptibility can be measured by carrying out a susceptibility test.

Table 3 shows the percentage of mosquitoes that experienced knockdown when exposed to 5 (five) types of exposure. Susceptibility tests were carried out on 2 (two) different groups, including a group of mosquitoes that grew from larvae without being exposed to citronella oil and a group of mosquitoes that grew from larvae that were exposed to 100µL/L citronella oil for 5 minutes. The percentage of mosquitoes in the group without treatment that experienced knockdown was more significant than those exposed to citronella oil, except for the 2.5% citronella oil treatment with 15-minute observations. Based on this, the response of mosquitoes in the group without exposure to lemongrass oil was more susceptible to the five types of exposure. The percentage of mosquitoes that experienced knockdown was analyzed by comparing the average obtained F_{count} 599.074, Sig value. 0.000 (<0.05) means there is a difference between the treatment groups, and the difference test continues. Based on table 4 shows that all treatment groups are significantly different from the PY control group (negative control). The 0.03% alphacypermethrin group (positive control) substantially differed from the 2.5% citronella oil group, with a mean difference in mosquito knockdown 5.8000 more significant than the 2.5% citronella oil group. Meanwhile, the 0.03% alphacypermethrin group had a mean difference in mosquito knockdown percentage of 6,2000, smaller than the combination group of 0.03% alphacypermethrin and 2.5% lemongrass oil. The difference test results did not show a fundamental difference between the 0.03% alphacypermethrin and combination groups.

The percentage of mosquito deaths after holding 24 hours shows no difference between the two groups of mosquitoes without treatment (A) and 100µL/L citronella oil (B), F_{count} 0.000, Sig. 1,000, significance value >0.05. So, exposure to 100µL/L citronella oil on instar III-IV mosquito larvae does not make mosquitoes more susceptible or more resistant to the insecticide alphacypermethrin 0.03%. The absorbance value analysis results using the T-test between the group without treatment and group B with 100µL/L citronella oil treatment showed a Sig. (2-tailed) 0.547>0.05 means there is no real difference between the two groups.

The acetylcholinesterase enzyme activity test was conducted on *Aedes aegypti* larvae and mosquitoes. This test is carried out by measuring the absorbance value (AV) using an ELISA reader at a wavelength of 415 nm. The absorbance value (AV) is linear with the activity of the acetylcholinesterase enzyme, where the more significant the absorbance value (AV), the greater the activity of the acetylcholinesterase enzyme. Resistance can be caused by the enzyme acetylcholinesterase (AChE) activity, which occurs when the enzyme blocks insecticidal compounds from reaching target organs. This AChE enzyme is one of the detoxification enzymes, where an increase in the AChE enzyme indicates the detoxification mechanism of insecticide metabolism in the insect's body. So, the enzyme acetylcholinesterase (AChE) as the target site for organophosphate class insecticides becomes a reference for causing resistance in mosquitoes (Selvi et al., 2010; WHO, 2007). The AChE incentive is a factor in the occurrence of resistance.

Various studies on natural ingredients that are referred to inhibit the activity of the acetylcholinesterase enzyme include research by Sriramya et al. (2017) on repellents from Chromolaena odorata, show that the stigmasterol and 1-hexacosanol content can inhibit the acetylcholinesterase activity of Aedes aegypti and C. Quinquefasciatus mosquitoes at the molecular level. Chellapandian et al. (2021) studied 25 types of alkaloids selected as ligands and their docking ability with acetylcholinesterase 1 in Aedes aegypti. The results show that alpha-solanine is the best type of alkaloid in the AchE1 binding pocket, with a minimum binding energy of -8.13kJ.mol. Other research by López and Pascual-Villalobos (2010) shows that most monoterpenoids tested inhibited the enzyme acetylcholinesterase; fenchone, S-carvone, and linalool produced the highest inhibition. Furthermore, it was observed that fenchone, γ-terpinene, geraniol, and linalool showed a reversible competitive inhibition, at least at the enzyme's hydrophobic active site. S-carvone, estragole, and camphor produced a mixed inhibition for this enzyme binding to either the free enzyme or the enzyme-substrate complex, which links to a different site from the active site where the substrate binds (López & Pascual-Villalobos, 2010). Studies of methanolic extracts and essential oils obtained from Asteraceae, Lamiaceae, Brassicaceae, and Amaryllidaceae were evaluated in vitro for AChE inhibitory activity. Essential oils of Origanum vulgare subsp. hirtum Ietswaart., Satureja pilosa Vel., Monarda fistulosa L., Thymus longedentatus (Degen & Urum.) Ronniger and the methanolic extract of Leucojum aestivum L. showed the most potent activity. Carvacrol was identified as the main component of the most active essential oils. In L. aestivum extract, galanthamine was found as the main alkaloid. The results indicate that essential oils and alkaloid-rich plant extracts possess the strongest AChE inhibitory activity. This gives us a reason to recommend that these plant products be tested for insecticidal activity in the future (Borislav et al., 2022).

CONCLUSION

Based on the results and discussion in this study, it can be concluded that the percentage of mosquito deaths after holding 24 hours, the results show there is no difference between the two groups of mosquitoes without treatment (A) and 100µL/L lemongrass oil (B), F_{count} 0.000, Sig. 1,000, significance value >0.05. Exposure to 100µL/L citronella oil on instar III-IV mosquito larvae did not make mosquitoes more susceptible or more resistant to the insecticide alphacypermethrin 0.03%. The absorbance value analysis results using the T-test between group A/without treatment and group B with 100µL/L lemongrass oil treatment showed a Sig. (2-tailed) 0.547>0.05 means there is no real difference between the two groups.

CREDIT AUTHOR STATEMENT

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